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Spectroscopic Properties and Redox Equilibria of Cytochrome *c* Oxidase

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SPECTROSCOPIC PROPERTIES AND REDOX EQUILIBRIA OF CYTOCHROME *c* OXIDASE

by

Boel Lanne

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Introduction

A person weighing 80 kg absorbs, at rest, 400 l of oxygen per day. There are several reactions consuming oxygen in higher organisms, but the predominant one is the last step in the so-called respiratory chain where cytochrome *c* oxidase catalyzes the reduction of oxygen to water. The conversion of a sugar to carbon dioxide and water in living organisms takes place in a series of at least 30 reaction steps. The reducing equivalents obtained in this oxidation process ultimately reach the respiratory chain, which is built up of approximately ten components all situated in or at the mitochondrial inner membrane. Three of the reactions in the respiratory chain (including the one catalyzed by cytochrome *c* oxidase) proceed with a large decrease in free energy, and this energy is used to drive the endergonic addition of a phosphate group to adenosine diphosphate. The adenosine triphosphate (ATP) formed in this way serves as a general energy carrier in the cell.

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase EC 1.9.3.1) has a relative molecular mass of approximately 200 000 and is thought to consist of seven different subunits. The functional unit contains two copper ions, Cu_A and Cu_B, and two heme components, called cytochrome *a* and a_3. The molecule spans the mitochondrial inner membrane. Detergents have to be used to solubilize the membrane and to separate cytochrome oxidase from the other firmly bound membrane components. A number of reviews treating cytochrome oxidase have recently been published [1-6].

The reaction catalyzed by cytochrome oxidase can be written:

$$4 \text{ cyt. } c \text{ (II)} + 4 H^+ + O_2 \rightleftharpoons 4 \text{ cyt. } c \text{ (III)} + 2 H_2O \quad (1)$$

Cytochrome *c* contains a single heme group and can consequently accept only one electron, while four electrons are needed to reduce dioxygen to water. As cytochrome oxidase contains four reversibly reducible metal ions, it might transfer more than one electron to oxygen at a time. There is, however, no satisfactory answer to the question how the metal ions receive, store and donate the electrons.

As seen in reaction (1) protons are consumed in the reduction of oxygen. It has also been shown [7,8] that protons are pumped across the mitochondrial inner membrane by cytochrome oxidase. The proton gradient produced in this way is thought to be used by a separate protein complex, often referred to as the mitochondrial ATPase, to drive the synthesis of ATP (for a recent discussion, see [9]).

EPR and optical spectroscopy are useful methods in studies of cytochrome oxidase because the heme irons and copper ions may show characteristic spectra that are influenced by the redox state and the immediate environment of the ions. EPR spectroscopy offers the advantage that one can determine the concentration of the species observed, which is not always the case in optical spectroscopy. This difference depends on that the transition probabilities between the energy levels studied in EPR spectroscopy can be obtained by quantum mechanical considerations, while the extinction coefficients used in optical spectroscopy have to be empirically determined.

The heme groups have several strong absorption bands in the visible part of the optical spectrum. Unfortunately, the bands of cytochrome *a* and a_3 overlap, and it is difficult to determine their relative contributions (see below). However, the problems of resolution is even larger for mitochondrial particles containing the other components of the respiratory chain. In this case both optical and EPR spectra are difficult to resolve. Cytochrome oxidase in mitochondrial particles cannot be obtained in concentrations high enough to allow thorough studies of all the EPR signals. Therefore, the increase in resolution and sensitivity are the main reasons for using isolated cytochrome oxidase in many types of studies.

I have restricted my studies to the spectral properties and redox equilibria of cytochrome oxidase. Knowledge obtained from such studies can provide important background for work aimed at understanding the details of the catalytic mechanism. In most studies described below cytochrome oxidase has been titrated anaerobically with ferrocytochrome *c*. Cytochrome *c* has a midpoint potential within the range of potentials spanned by the four metal ions in cytochrome oxidase. This means that the potential of the solution can be estimated from the redox state of cytochrome *c*. On the other hand, it is not possible to reduce cytochrome oxidase completely with ferrocytochrome *c*.

This thesis includes investigations of the EPR parameters of oxidized and partially reduced cytochrome oxidase, studies of the enzyme in the presence of the redox mediator hexacyanoferrate, and studies of the spectroscopic and redox properties at varying pH values. Cytochrome oxidase in nonionic detergent solution and in phospholipid vesicles have been compared and an

The heme groups have several strong absorption bands in the visible part of the optical spectrum. Unfortunately, the bands of cytochrome a and a_3 overlap, and it is difficult to determine their relative contributions (see below). However, the problems of resolution is even larger for mitochondrial particles containing the other components of the respiratory chain. In this case both optical and EPR spectra are difficult to resolve. Cytochrome oxidase in mitochondrial particles cannot be obtained in concentrations high enough to allow thorough studies of all the EPR signals. Therefore, the increase in resolution and sensitivity are the main reasons for using isolated cytochrome oxidase in many types of studies.

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analysis of redox data with the possibility of coupling between all four electron acceptors is presented. These studies are described in detail in the following papers:

I R. Aasa, S.P.J. Albracht, K.-E. Falk, B. Lanne and T. Vänngård: EPR Signals from Cytochrome *c* Oxidase, (1976) Biochim. Biophys. Acta 422, 260-272.

II B. Lanne, B.G. Malmström and T. Vänngård: Effects of Hexacyanoferrate on Cytochrome *c* Oxidase, (1977) FEBS Lett. 77, 146-150.

III B. Lanne and T. Vänngård: Redox Titrations of Cytochrome *c* Oxidase. An analysis of a multi-electron system, (1978) Biochim. Biophys. Acta 501, 449-457.

IV B. Karlsson, B. Lanne, B.G. Malmström, G. Berg and R. Ekholm: Spectroscopic and Catalytic Properties of Cytochrome *c* Oxidase in Phospholipid Vesicles, (1977) FEBS Lett. 84, 291-295.

V B. Lanne, B.G. Malmström and T. Vänngård: The Influence of pH on the EPR and Redox Properties of Cytochrome *c* Oxidase in Detergent Solution and in Phospholipid Vesicles, (1978), submitted to Biochim. Biophys. Acta.

EPR signals in oxidized cytochrome c oxidase

An EPR spectrum of oxidized cytochrome oxidase at 9.1 GHz is shown in Fig. 1a. Two major species can be observed, one at 0.21, 0.3 and 0.45 T and the other one around 0.32 T. The former signal is identified as a low-spin heme iron. The *g*-values of this signal are sensitive to a number of factors [10], including pH (paper V). At pH 7.4, which is often used

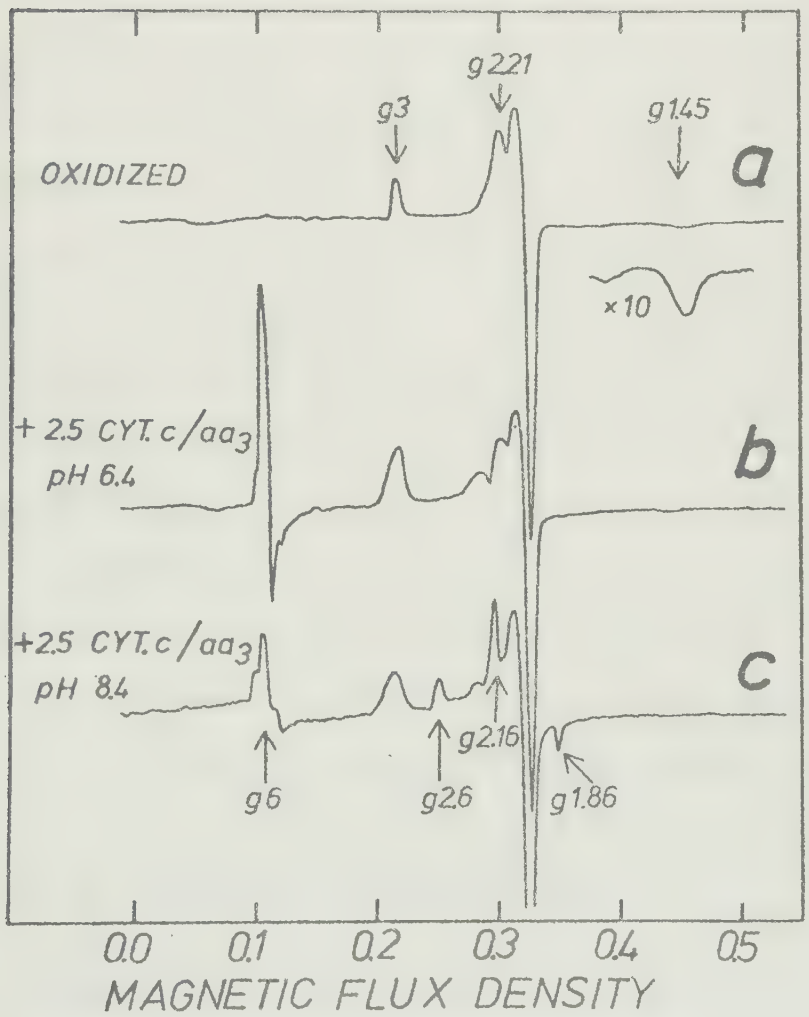

Fig. 1. EPR spectra of oxidized and partially reduced cytochrome oxidase.

in studies of cytochrome oxidase, the g-values are 3.03, 2.21 and 1.45 (paper I), but when the pH is decreased to 6.4 the g-values become 3.00, 2.21 and 1.49 (paper V). A few years ago it was pointed out that an incorrect formula had been used in earlier simulations of anisotropic EPR signals [11]. Using the correct formula ([12] and paper I) it is shown that the low-spin heme signal corresponds to one heme iron per functional unit of enzyme. This heme is usually assumed to be cytochrome a [3,13-16].

The origin of the large signal at 0.32 T has been debated (for a discussion, see paper I and [14]). The most favoured interpretation is that it originates from Cu(II). The signal has been found to consist of three species (paper I). One of these can be extracted by dialysis against EDTA. Since this treatment does not change the activity of the enzyme [17], this copper is thought to be an impurity. When the functional copper is studied at 35 GHz, it is found (paper I) to consist of two components. One of them has a more narrow $g_{\parallel}$ line and is reduced more easily with cytochrome c. Integrations show that the sum of the two firmly bound copper forms, Cu_A, amounts to between 0.7 and 0.9 per functional unit (paper I and [12]). There is at present no good explanation for the finding of this non-integral number.

In summary only two of the four metal ions can be accounted for in the oxidized enzyme. This may seem surprising, as reductive titrations [12,18,19] show that the enzyme can accept four electrons, which indicate that the metals are in the Cu(II) and Fe(III) states and should, in principle, give rise to EPR

signals. However, if the two missing ions, cytochrome a_3 and Cu_B, form a magnetically coupled pair [13,20,21] they would be EPR undetectable.

EPR signals in the partially reduced cytochrome c oxidase

If cytochrome oxidase is titrated anaerobically with ferrocytochrome *c*, the low-spin heme (*g* 3, 2.2, 1.4) and the copper (*g*2) signals decrease, while high-spin heme signals and, under some circumstances, new low-spin heme signals (*g* 2.6, 2.16, 1.86) appear (Fig. 1b and c). The broad *g*3 signal seen in Fig. 1b and c is due to oxidized cytochrome *c*.

The parameters of the high-spin heme signals are influenced by the milieu (paper V). In half-reduced enzyme at a neutral or alkaline pH one observes three components, two rhombic (*g* 6.42, 5.37, 2.0 and *g* 6.27, 5.49, 2.0) and one nearly axial (*g* 5.99, 5.79, 2.0) (paper I, see also Fig. 1c). The relative amounts of the three components depend on the degree of reduction of the enzyme, but are also seen to differ slightly between different preparations. The two rhombic high-spin heme signals disappear when the pH is decreased and a new, nearly axial component is formed (Fig. 1b). The ratio between the two nearly axial components present at low pH changes as the enzyme becomes more reduced (paper V).

The low-spin heme signals at *g*2.6 appear under the same conditions as the rhombic high-spin signals (paper V). When cytochrome oxidase is reduced with less than approximately one ferrocytochrome *c*, the *g*-values are 2.59, 2.16, 1.86.

With more reductant added this signal splits into two (*g* 2.57, 2.16, 1.86 and *g* 2.61, 2.16, 1.86). As the titration proceeds, more of the former signal is produced (paper V). These signals have earlier been found in very low amounts ([10] and paper I) and, therefore, not gained much attention. In paper V, it is, however, shown that the *g*2.6 signals correspond to maximally 0.35 heme per functional unit at pH 8.4.

Two main hypotheses concerning the origin of the *g*6 signals are currently discussed. The high-spin heme signals could come from those molecules where cytochrome a_3 is oxidized and Cu_B reduced [13], or from cytochrome *a* changing state from a low-spin form when cytochrome a_3 is reduced [16]. For the moment I regard the first hypothesis to be the most probable (for a discussion, see paper III). It has also been suggested that the rhombic and axial *g*6 signals originate from different hemes [6,14]. In paper V it is also proposed that cytochrome a_3 shifts from high spin to low spin when the pH is increased, thereby giving rise to the *g*2.6 signals.

Redox titrations of cytochrome oxidase

The literature on the redox properties of cytochrome oxidase does not present a clear picture. Both the experimental results and the interpretations differ widely. Most redox titrations have been performed using optical spectroscopy. Both hemes are considered to contribute to the absorption bands appearing at 605 (α-band) and 445 nm (γ-band) on reduction of the enzyme (Fig. 1 in paper II). One of the copper ions, Cu_A, is often assumed to be responsible for the whole

absorbance change at 830 nm (however, see [22,23]). To analyze such optical titrations one must know what the relative spectral contributions of the different species are and if there is any spectral interaction in the sense that the redox state of one species would have an effect on the optical properties of the other titrating species (cf. paper III). As an illustration to the difficulties encountered in redox titrations the results from studies of the α-band will be discussed in some detail.

Most of the optical titrations have been published by three groups. Two of them, led by B.F. van Gelder and D.F. Wilson, respectively, follow the absorbance change as a function of the potential of the solution and extract the spectral contributions and the redox potentials of the two hemes. The van Gelder group [18] arrives at the conclusion that both hemes have almost the same absorbance change on reduction, 45% and 55%, and that their redox potentials are +370 and +230 mV. Wilson´s group on the other hand [24], finds a high potential heme, $E^{o'}$ = +360 mV, that contributes with 35% and a low potential heme, $E^{o'}$ = +205 mV, that contributes with 65% of the absorption change at this wavelength. Hartzell and coworkers monitor the amount of electrons accepted or donated by cytochrome oxidase [19,25]. In favourable cases such experiments enable determinations of the redox potentials of all electron acceptors in the enzyme, even those that do not absorb at the wavelength studied. For many years these workers held the view that the two hemes contribute equally to the α-band (see for example [26]), but now they claim that the high potential

heme contributes with only 20% of the absorbance change [27]. The obtained redox potentials are +340 mV for one heme and one copper, +240 mV for the second copper and +220 mV for the second heme. It is now generally accepted that some of the acceptors in cytochrome oxidase have pH-dependent redox potentials([12,28,29] and paper V), and it is therefore unfortunate that the Hartzell group in 1977 changed the pH of their experiments from 7.0 [19,26] to 7.6 [27]. The optical titrations described above were originally analyzed without any optical or potential interactions although studies with inhibitors (see, for example [30]) and redox titrations followed by EPR spectroscopy indicate such interactions (see below).

Few titrations of isolated cytochrome oxidase followed by EPR spectroscopy have been made. Hartzell and Beinert [12] made reductive titrations using either NADH plus phenazine methosulfate or ferrocytochrome *c* as reductants. They found that the low-spin heme signal ($g3$) and the copper ($g2$) have midpoint potentials of +340 and +260 mV, respectively. The appearance and disappearance of the high-spin heme signal occur at +345 and +210 mV, respectively. These figures cannot be taken directly as the midpoint potentials of the EPR invisible copper, Cu_B, and the low potential heme, as done by Palmer *et al.*([31], see also [12]). With these redox potentials the $g6$ signals should correspond to 0.8 heme per functional unit at partial reduction (paper III), but only 0.25 to 0.5 is actually seen ([12], paper I and V). This dilemma will be discussed below (see also paper III).

Different authors have used different conditions in their redox titrations with respect to, for example, buffer composition, pH, ionic strength, detergent, reductant/oxidant and redox mediators. Variations of some of these conditions have been found to have quite a marked influence on the redox behaviour of cytochrome oxidase. We have studied the effects of the redox mediator hexacyanoferrate (paper II) and of the changes of hydrophobic milieu (paper IV) and pH (paper V).

The effects of hexacyanoferrate on cytochrome oxidase

Hexacyanoferrate is a frequently used redox mediator in studies of cytochrome oxidase [12,18,19,29]. We found some unexpected effects with hexacyanoferrate(II)/(III) as a redox buffer. Anaerobic incubation of enzyme and mediator result in a fairly rapid reduction, judged from both optical and EPR spectra. This is followed by small decreases of the absorbances at the α- and γ-bands (Fig. 1 in paper II) and a marked decrease of the *g*6 signals. These changes continue for at least one hour at room temperature. Using ferrocytochrome *c* as reductant instead, equilibrium is reached within 20 min, and the maximal amount of *g*6 signal obtained is two to four times larger (the same buffer and pH were used in these experiments, cf. below). In addition, the EPR signal from hexacyanoferrate(III) formed on anaerobic mixing of cytochrome oxidase and hexacyanoferrate(II) is more narrow and slightly shifted as compared to the signal obtained on mixing hexacyanoferrate(III) and enzyme aerobically (Fig. 3 in paper II). This is interpreted as a bidning of hexacyano-

ferrate to the partially reduced enzyme. Optical titrations using hexacyanoferrate(II) or ferrocytochrome *c* as reductants are presented (Fig. 2 in paper II). These indicate that differences in the redox behaviour depend upon the reductant used. With our present knowledge concerning pH effects on the redox potentials, part of these differences can be explained by these two titrations not being carried out at the same pH. Considering our results and a report [32], in which hexacyanoferrate was shown to inhibit the oxidase, we decided to continue to titrate cytochrome oxidase with its natural substrate, ferrocytochrome *c*.

Properties of cytochrome oxidase in phospholipid vesicles

The natural environment for cytochrome oxidase is a phospholipid bilayer (see *Introduction*). In the isolated form the enzyme contains varying amounts of phospholipids which might explain the difficulties in obtaining reproducible results and possibly of the variation in some of the EPR signals. It is also known that the activity of the isolated enzyme increases when phospholipids are added [1]. With this in mind, we decided to study the EPR properties of vesicle oxidase (paper IV and V).

We used egg phosphatidylcholine vesicles and controlled that the enzyme had been incorporated by electron microscopy, activity measurements, and nuclear magnetic resonance spectroscopy. The EPR spectrum of oxidized vesicle oxidase is very similar to that of a good preparation in nonionic detergent (Fig. 3 in paper IV). The high-spin heme signals appearing on

partial reduction with ferrocytochrome *c*, on the other hand, do not contain the rhombic components observed in detergent oxidase at the same pH, but consist of two nearly axial components (paper I and IV). In addition, the *g*2.6 signals are totally absent. These observations were first interpreted as an improvement in homogeneity (paper IV). A slightly altered interpretation emerged when this system was studied further (see next section).

Effects of pH on the redox properties of cytochrome oxidase

It is pointed out above (pages 5-7) that the pH affects the EPR parameters of the heme signals in both the oxidized and partially reduced enzyme. In paper V it is concluded that there is at least one titrating group in the vicinity of the hemes and that the titration of this group influences both hemes, changing the *g*-values of cytochrome *a* and inducing a high-to-low spin shift in cytochrome a_3. This shift is suggested to depend on a change of a liganded H_2O to OH^-.

When cytochrome oxidase is titrated with ferrocytochrome *c*, fewer equivalents of the reductant are needed to reduce cytochrome *a* as the pH is increased (Fig. 4 in paper V). The reduction of Cu_A also changes in the same direction but less markedly. As pointed out in paper V, these results are essentially in accordance with those obtained by Hartzell and Beinert [12]. The maximal amount of *g*6 signals obtained decreases from 0.5 to 0.1 heme per functional unit, and the amount of *g*2.6 signal increase from zero to 0.3 heme when

the pH is changed from 6.4 to 8.4. Thus, the sum of the two heme signals of the partially reduced enzyme is not much changed in the pH interval studied.

The redox behaviour of vesicle oxidase at different pH values is compared to that of oxidase in nonionic detergent. It is then found that the same reduction in the number of different EPR signals as is obtained on incorporation of oxidase into vesicles, can also be obtained by simply decreasing the pH of the oxidase in detergent solution. The following changes occur on both decreasing the pH and incorporation into vesicles: the *g*-values of the low-spin signal in oxidized enzyme move towards 2, the *g*2.6 signal decreases, the shape of the *g*6 signals changes to the low-pH type, and the catalytic activity increases.

Analysis of multi-electron systems

To describe fully the redox equilibria of the 16 possible molecular forms of a 4-electron system, 15 equilibrium constants are needed. They can be divided into 4 intrinsic constants, 6 for pairwise interactions, 4 describing interaction between 3 acceptors at a time and 1 for the fourth order interaction. In paper III only pairwise interactions are considered which, together with the intrinsic constants, gives 10 parameters. For every physical property studied, e.g. optical spectroscopy at a specified wavelength, an EPR signal or the bulk magnetic susceptibility, 10 new experimental parameters can be used in addition to the 10 potential constants. Redox titrations are usually analyzed without any interactions, i.e. with 4 parameters describing

the redox potentials of the enzyme and 1 parameter for the absorbance change at for example 605 nm [18,19,24, 26-29]. Some theoretical treatments of published data [3,6,33] including potential interaction between the two hemes (5 potential parameters) have been made, but such a model is not enough to explain EPR data. However, with the model presented in paper III, the EPR titrations of Hartzell and Beinert [12] can be simulated very well (Figs. 1 and 2 in paper III), including the observation that the *g*6 signals appear early and disappear late in a reductive titration and that still only low amounts of them are formed (cf. page 10). This fit is obtained when three acceptors (the two hemes and Cu_B) are involved in such pairwise potential interaction that they titrate over a large potential interval. However, no unique solution to the EPR titrations could be given. This lack of uniqueness is even more obvious when one examines optical titrations, since more than one acceptor absorbs at most wavelengths and, in principle, the extinction coefficients of the acceptors are unknown. This means that the redox potentials and relative contributions given in the literature for titrations of, for example the α-band, represent just one of the many possible solutions to a titration curve.

Concluding remarks

Despite the fact that cytochrome oxidase has been studied for fifty years we are still far from a full comprehension of the mechanistic function of the enzyme. Most work on the kinetic and equilibrium properties of cytochrome oxidase

have been analyzed in terms of the enzyme being a non-interacting four-electron acceptor. However, both optical and potential interactions have to be assumed to explain most data obtained by EPR spectroscopy and work with inhibitors. The many signals seen in EPR spectra have been suggested to depend on heterogeneities in the enzyme, but some of them could, for example, be due to the presence of different conformations in equilibrium with each other (cf. the pH-dependent high-to-low spin transformations). The existence of different conformations of cytochrome oxidase and of interactions between the electron acceptors will make the analysis of experimental data tedious, but this battle must be fought if one wants to understand the details of the catalytic mechanism of cytochrome oxidase.

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